Formation of Subcuticular Coralloid Hyphae by *Phomopsis leptostromiformis* Upon Latent Infection of Narrow-Leafed Lupins

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ABSTRACT

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The events leading to latent infection of narrow-leafed lupins (*Lupinus angustifolius*) by *Phomopsis leptostromiformis* were examined by light microscopy. One-centimeter stem segments of 28-day-old plants of cultivar Yandee were inoculated with a suspension of 1×10^6 conidia ml^{-1} and kept under high humidity for 48 hr. Stem segments were excised, and the epidermis was examined after stripping a three-cell layer from the subepidermal tissue at 48, 60, 72, 84, and 96 hr and at 5, 6, 7, 8, 9, 10, and 20 days after inoculation. Germination of conidia was 100% on nutrient agar, but germ tubes did not develop when inoculated onto stems in water suspensions. On stems, germination could be observed only as a stained attachment to the cuticle in 97% of conidia. Four days after inoculation, penetration of the cuticle was observed through this attachment directly beneath the conidia, without the formation of an appressorium. Penetration was arrested between the cuticle and the epidermis, where a distinctive coralloid mycelium was visible by 7 days after inoculation. At 20 days, these coralloid structures were present at a mean frequency of 148/cm^2 with a mean length of 192 μm and mean width of 101 μm. Plants remained symptomless 20 days after inoculation. Germination of conidia was also 97% on stems of resistant breeding line 75A258. However, on 75A258, the coralloid infection structures were rare (0.8/cm^2), and all were less than 10 μm in length and width. These coralloid structures were observed occasionally in symptomless green plants from the field and were present on mature stems coincident with symptoms of Phomopsis stem blight. Normal mycelia were observed to invade the subepidermal tissue of senescent stems from the coralloid hyphae. This is the first known report of a coralloid subcuticular infection structure for *Phomopsis* spp. on any host plant.

Phomopsis stem blight of lupins (lupines, *Lupinus*) (9) has many features of a latent disease (20), but until now, the latent infection structure of *Phomopsis leptostromiformis* (Kühn) Bubák (teleomorph *Diaporthe woodii* Punithalingam) has not been reported. Infectious inoculum of the fungus was shown to be present throughout the growing season, and narrow-leafed lupin (blue lupine, *L. angustifolius* L.) plants became infected at all ages. However, symptoms of Phomopsis stem blight did not normally appear until the plants matured and began senescing at the end of the growing season (18). Premature killing of plants with paraquat-diquat herbicide (7) showed that infection of *L. angustifolius* by *P. leptostromiformis* occurred within 48 hr of inoculation. Previous studies (8,13) have been conducted into the early stages of infection on various lupin species; however, none of these have reported the mode of penetration or the means by which the fungus survives in symptomless plants as a latent infection.

Resistance breeding programs have been hampered by the lack of a suitable greenhouse infection test for quantifying the extent of latent infection. For example, only one assessment of resistance to Phomopsis stem blight in *L. angustifolius* may be made each year in the field, and these ratings are subject to genotype × environment interactions (5). Such reliance on field assessment of resistance has made evaluation of the genetics of resistance time-consuming and inconclusive (3). Moderate resistance to Phomopsis stem blight reduces lupinosis toxicity of lupin stubble (4), but greater resistance levels are needed in commercial varieties.

We have developed a method of detecting and quantifying latent infection of lupins by *P. leptostromiformis*. A staining procedure was developed to observe subcuticular latent hyphae that were not detected in previous studies. The procedure may be useful in screening lupins for resistance to Phomopsis stem blight, in detecting variations in relative virulence in the pathogen population, and in determining the environmental conditions for infection. A preliminary report of this work has been published (17).

MATERIALS AND METHODS

Inoculation and observation of susceptible lupins. A cultivar of narrow-leafed

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lupin (Yandee) that is susceptible to *P. leptostromiformis* was grown in a glasshouse (20 C day/15 C night) in five 190-mm-diameter plastic pots containing clean white sand (not autoclaved). Nine seeds were planted per pot and seedlings were thinned to seven per pot after germination. Plants were grown for 28 days before inoculation of the plants in four pots; plants in the fifth pot were controls and sprayed with water only.

An isolate of *P. leptostromiformis* was obtained from excised tissue of lupin (cv. Danja) stubble from the Lake Magenta area of Western Australia and grown initially on potato-dextrose agar (PDA) + Aureomycin (chlortetracycline hydrochloride) (2%) at 25 C for 5 days. Subcultures were placed on PDA for a further 5 days, and small portions of the colony were stored in 15% glycerol in small containers at −20 C. Inoculum consisting of pycnidioconidia was produced by subculturing the isolate onto oat agar for 10 days at 25 C. The colonies were transferred to a cabinet with near-UV light alternating 12-hr light/dark cycles for a further 4 wk to induce the formation of fertile pycnidia. Conidial suspensions were prepared by flooding plates with 5 ml of sterile distilled water and gently removing globules of conidia from the pycnidia with a Pasteur pipet. Viability of conidia was assessed on PDA. Inoculum was applied by spraying stems of seedlings between the first and second leaf nodes with a suspension of 1×10^6 conidia ml^{-1} of distilled water using an artists' air brush.

After inoculation, the pots were placed within clear plastic bags and shaded from direct sunlight for 48 hr. Two plants were sampled for microscopic examination and photography at 48, 60, 72, 84, and 96 hr and at 5, 6, 7, 8, 9, and 10 days after inoculation, and six plants were examined 20 days after inoculation. Control plants were examined at every second time of sampling. This experiment was repeated several times to confirm the microscopic observations on Yandee.

Microscopic examination of inoculated stem tissue. One-centimeter stem segments from the inoculation site were boiled in water for 60 s, and the epidermal tissue was carefully removed with a scalpel and forceps from the entire circumference of the stem. Using this technique, three-cell layers, including the epidermis, were usually recovered. The epidermal tissue was cleared and stained in alcoholic lactophenol cotton blue following the method of Shipton and Brown (11) and mounted on a microscope slide in 50% glycerol. The infection process was observed under the microscope and photographed at each time of sampling. Germination of conidia was assessed 48 hr after inoculation as the frequency of conidia with stain-absorbing attachments to the cuticle. Penetration of the cuticle and depth of penetration of the hyphae were observed by folding a piece of epidermal tissue in half, mounting under a coverslip, and rolling the tissue until the subcuticular hyphae could be viewed at the fold.

Assessment of resistance. Susceptible cultivar Yandee and a resistant breeding line, 75A258, were grown and inoculated using the techniques described earlier with the exception that there were two pots of each variety and plants were thinned to five per pot after germination. Germination of conidia and the frequency and size of coralloid latent infection structures were measured on all plants 20 days after inoculation.

Microscopic observation of field-infected lupins. Ten to 20 plants of commercial cultivars or weed populations of *L. angustifolius*, *L. albus* L., and *L. luteus* L. were collected throughout the growing season (May–October) from the field in Western Australia and examined in the laboratory for the presence of infection structures of *P. leptostromiformis* in stem tissue. Stubble of narrow-leafed lupin crops was also collected and examined for latent and colonizing hyphae in tissue displaying symptoms of Phomopsis stem blight.

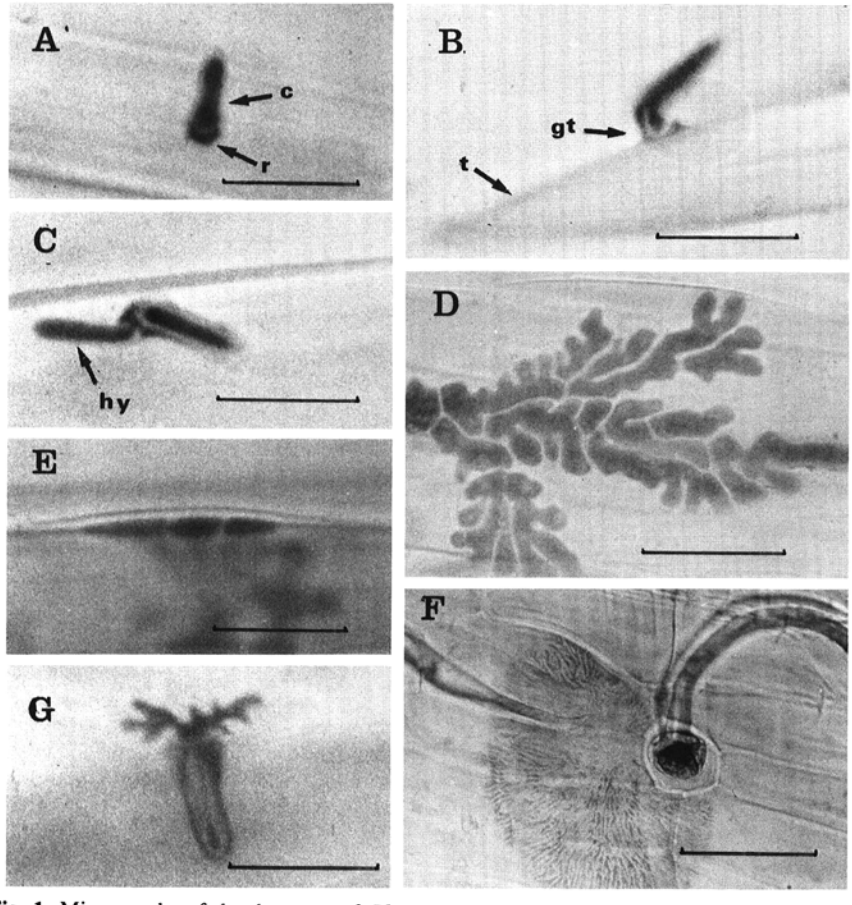

Fig. 1. Micrographs of development of *Phomopsis leptostromiformis* on stems of lupin. **(A)** Germinated conidium (c) of *P. leptostromiformis* showing thickened ring (r) when viewed from directly above at 48 hr. Bar = 10 μm. **(B)** Side view of conidium showing the swelling at the attachment of germtube (gt) to stem trichome (t) at 48 hr. Bar = 10 μm. **(C)** Conidium of *P. leptostromiformis* with direct penetration of the cuticle and the initial formation of subcuticular hyphae (hy) at 96 hr. Bar = 10 μm. **(D)** Coralloid hyphal structures 7 days after inoculation of stems of susceptible lupin cv. Yandee. Bar = 10 μm. **(E)** Folded epidermal tissue showing the position of the hyphae lying below the cuticle at 7 days. Bar = 10 μm. **(F and G)** Coralloid hyphal structures 20 days after inoculation on cv. Yandee and resistant breeding line 75A258. Bar = 100 and 10 μm, respectively.

RESULTS

Infection of susceptible lupins. Viability of inoculum of *P. leptostromiformis* was 100% on PDA and conidia produced rapidly growing germ tubes. On stems of Yandee, 97% of conidia of *P. leptostromiformis* were observed to have germinated by 48 hr after inoculation, but germ tube growth was suppressed and could be observed only as a dark stain-absorbing ring at one end of the conidium (Fig. 1A). A side view showed this ring to be a thickened substance of unknown composition at the point where the germ tube was attached to the cuticle (Fig. 1B). This attachment of the germ tube to the cuticle was very firm, and conidia were not dislodged by vigorous boiling in an alcoholic lactophenol cotton blue stain. The number of conidia remaining on the stem surface after staining was calculated to be $1 \times 10^5/cm^2$. Penetration was not observed

until 4 days after inoculation. Directly beneath the conidium at the point of attachment to the stem, a narrow infection peg pierced the cuticle but did not enter the epidermis. Dark-staining hyphae developed between the epidermis and the cuticle (Fig. 1C).

Seven days after inoculation, the subcuticular hyphae had formed a distinctive "coralloid" shape up to 50 mm in length (Fig. 1D). The depth of penetration of the hyphae was determined by viewing the edge of a piece of epidermal tissue folded across the plane of the coralloid hyphae. The bulge of the cuticle over the growing hyphae can be distinctly seen in Figure 1E. Many conidia were observed to be attached to trichomes on the stem with penetration and development of subcuticular hyphae similar to that observed on the stem surface. No subcuticular hyphae were observed entering the basal hair cell after penetration through the trichome cuticle. *P. leptostromiformis* was reisolated only from the inoculated sites, and the coralloid hyphae were absent from control plants.

Comparison of resistant and susceptible lupins. Twenty days after inoculation of susceptible Yandee, the coralloid structures were present at a mean frequency of $148 \pm 40/cm^2$. The dimensions of the structures ranged from 25 to 300 μm in length and 25 to 230 μm in width with means of 192 ± 73 μm and 101 ± 63 μm, respectively (Fig. 1F). Coralloid hyphae were often observed aligned along the middle lamella of the epidermal cells. The hyphae were often broad (2–10 μm) with nonstained septa between cells. On resistant breeding line 75A258, the structures were rare or absent at 20 days (mean $0.8 \pm 1/cm^2$) and when present were not longer than 10 μm (Fig. 1G). The hyphae appeared to be narrower (<1 μm wide) and often had a suppressed "staghorn" appearance. The number of conidia per unit area of stem tissue after staining ($10^5/cm^2$) and the percent germination (97%) was not significantly different between Yandee and 75A258.

Microscopic observations of field-infected lupins. The coralloid structures were observed occasionally in symptomless plants from the field. Two observations were made on *L. angustifolius* cv. Yandee and one each on cv. Danja and a weed population of *L. luteus*. No structures were observed on *L. albus*. Excised epidermal tissue from lesions of Phomopsis stem blight on lupin stubble collected after seed harvest also revealed the presence of subcuticular coralloid hyphae with a proliferation of normal mycelia invading the subepidermal tissue from the coralloid hyphae.

DISCUSSION

The present study reports for the first time the existence of subcuticular coralloid latent hyphae in lupin stems infected with *P. leptostromiformis*. Conidia of *P. leptostromiformis* do not form long germ tubes on narrow-leafed lupin stems, but germination is accompanied by the formation of a stain-absorbing swelling at the point of attachment to the cuticle. Penetration of the cuticle occurs directly below conidia through this thickened point of attachment. This unusual behavior explains in part why previous workers (8,13) have failed to detect the latent infection structure. It is difficult to observe the structure unless a thin layer of epidermal tissue is removed and stained as we have described. It is also important to inoculate plants with suspensions of conidia in nutrient-free water—inoculation in (2%) gelatin induced the formation of long germ tubes and coralloid hyphae were not observed (*data not shown*).

Our field observations indicate that coralloid hyphae are rare in field-infected plants, but this is not surprising as not all plants show symptoms of Phomopsis stem blight at the end of the growing season, and some environments result in very little disease at all (4–6). However, the observations on stubble provide the first evidence that normal colonizing hyphae grow directly from coralloid hyphae and invade the dead stem tissue upon senescence.

A number of plant pathogens are known to establish subcuticular latent hyphae after penetration. Most of the early work on subcuticular hyphae was carried out on ripe rot of fruits and was reviewed by Verhoeff (15). Information of such structures with stem and foliar infections is rare. *Rhynchosporium secalis* (Oudem.) J. J.Davis (2), *Venturia inaequalis* (Cooke) G. Wint. (10), *Botryosphaeria vaccinii* (Shear) Barr (16), and *Alternaria brassicae* (Berk.) Sacc. (12) all form subcuticular hyphae before further colonization of leaves of their respective hosts. All of these fungal pathogens have a relatively short latent period. *P. leptostromiformis* has a long latent period in narrow-leafed lupins and does not normally cause symptoms on living plants. As a colonizer of senescing and dead plant tissues, it produces a toxin that causes lupinosis in grazing animals (1,14,19).

The lack of symptoms during this long latent period has made breeding for resistance a long-term project dependent on annual field results. Selection for resistance is currently made by visual assessment of lesions of Phomopsis stem blight on senescent plants in the field (5), followed by assays of infested stubble for the lupinosis hepatotoxin (4). Breeding for resistance has achieved success so far in reducing toxicity of stubble (4) and reducing pod and seed infection (6), but there is a need for higher levels of resistance with further reductions in stem toxicity and seed infection.

The discovery of the latent infection structures of *P. leptostromiformis* makes it possible to intensify research into host resistance, pathogen relative virulence, and environmental parameters associated with infection. The techniques used in this study could be refined to establish a relatively quick method of measuring resistance for large-scale screening in the greenhouse during early generations of the breeding program. Our preliminary results indicate that resistance is strongly associated with reduced formation of coralloid hyphae; this should be tested using a range of genotypes with different sources and levels of resistance.

The formation of the subcuticular coralloid hyphae after infection by *P. leptostromiformis* is considered to be a new and interesting phenomenon. We are not aware of any earlier report of such coralloid subcuticular infection structures in the genus *Phomopsis*.

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